SHEET-LIKE DIAGNOSTIC DEVICE

The invention relates to a solid diagnostic device which comprises several functional sectors and is used for the detection and quantitative determination of substances or analytes in biological fluids. The invention also relates to a process using this device in which, after the device has come into contact with the fluid, the analytes react with specific combination partners having biological affinity and are detected by means of labelling reagents.

In methods of diagnosis, the ability to identify and estimate specific compounds has made it possible to monitor the administration of medicaments, the quantification of physiologically active compounds or secondary products thereof and the diagnosis of infections. In this respect, the immunoassay methods (RIA, ELSIA and the agglutination test) are of particular importance. The specific combination reactions utilized in the tests are not limited to immunological interactions, such as antigen-antibody or hapten-antibody interactions, but also utilize interactions having biological affinity, such as lectin-sugar or active compound-receptor.

Although the existing tests are sensitive and specific, they do not constitute convenient application forms, because of the long duration of the test (in most cases several hours or even days) and the frequent test steps, such as immune reaction, washing steps and enzymatic reaction. The long test times are not compatible with use in emergency methods of diagnosis.

Integrated dry chemical test elements, such as are described in the present invention, simplify the performance of the tests and shorten the test times.

No sheet-like test element, in which all the components of the immune reaction of a heterogeneous immunoassay using solid phase detection, and the functional performance and the "bound-free" separation, are integrated has been described so far.

Whereas in the test strip assembly the immune reaction steps and the separation of bound and free phases are operated in the heterogeneous test by directed streams of liquid, in test element assemblies operating by means of thin layers laminated over one another (film technology), processes controlled by diffusion and directed by the concentration gradient are possible driving forces. A fluorescence labelling is used in German Offenlegungsschrift No. 3,329,728 (Japanese Patent No. P144,341/82) and EP A No. 0,097,952 (Japanese Patent No. 114,359/82). The labelling has a low molecular weight and hence promotes processes controlled by diffusion. However, the test has to be carried out at an elevated temperature. In the first of these two cases both the free phase and also the bound phase are evaluated. In film technology the absorption of solvent is effected either by hydrating swellable components or by filling capillary cavities. In the case of assemblies having layers laminated over one another only the top layer and the bottom layer are accessible to detection without major difficulties.

After the reaction steps have taken place it is difficult to react reagents with components in intermediately placed layers. In the test strip assembly having zones situated one behind another, such as is used in the present invention, in principle each zone is readily accessible, both from above and also from below, for a determination and also for the addition of reagents which may perhaps be required.

The invention relates to a sheet-like diagnostic device which contains all the reagent components and which contains not only all the components required for the functional sequence, but also the functional sequences themselves in an integrated form, and by means of which it is possible to detect an analyte having properties of biological affinity, in such a way that a solution of the analyte is brought into contact with a functional region of the device designed for this purpose, and the analyte as detected via a signal-producing system in a single functional region, a solid phase zone.

A second analyte, or further analytes, as constituents of the same solution can be detected at the same time by means of the device, if these analytes possess properties of biological affinity different from the first analyte. They are also detected in the same manner as the first analyte in a single functional region, a solid phase zone appropriate for them. The functional regions for the detection of the second or further analytes are situated on the sheet-like device in front of or behind the functional region for the detection of the first analyte. The device can also contain several solid phase zones which are appropriate for an analyte and different measurement ranges of this analyte. The device contains all reactants and reagents in a dehydrated form.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is a summary table illustrating test assemblies according to certain preferred embodiments of the present invention; and

FIG. 2 is a summary table illustrating test assemblies according to certain preferred embodiments of the present invention.

DESCRIPTION OF PREFERRED EMBODIMENTS

The sheet-like diagnostic device comprises one or several strips, arranged behind one another, of material which have a capacity for absorbing aqueous solutions. The strips are fixed on a solid support. They contain the reagent components required for the particular diagnostic agent and thus become functional sectors or functional regions. The functional sector situated at one end of the strip-shaped device (solvent application zone) is brought into contact with the analyte solution by being dipped into the latter or by the application of the latter. The solution migrates through all the functional regions. The absorptive capacity of the supporting materials of which the strips are composed causes a flow of liquid which stops at the other end of the strip-shaped device. The analyte can also be applied in the middle region of the device, and a flow of liquid from one end of the device to the other can then be induced.

The sample does not have to be applied directly to the chromatographing section of the device. It can also be applied to an absorptive material which is situated on the device and has the function of removing blood cells from the sample. After being filtered the sample then reaches the device. In the course of this filtration process the addition of reagents can be effected at the same time by dissolving the latter out of components present in the filter in a dry state. Interfering factors can be eliminated from the solution by means of such components. Thus, for instance, the ascorbic acid present in a sample, which interferes in the use of oxidases and peroxidases as labelling agents, can be rendered harmless by means of a suitable oxidizing agent. Furthermore, the filter can also have the function of an adsor-

bent which removes interfering factors from the sample by adsorption. The filtration, adsorption and reagent admixing function for conditioning the sample for the test can also be taken over by the mobile phase application zone or a zone situated behind the latter.

The distribution of the solvent in the individual functional regions depends on the adsorptive capacity and the dimensions of the materials used.

The solvent application zone can have the function of a volume metering element, as described in German Patent Nos. 3,043,608 and 2,332,760, and U.S. Pat. Nos. 3,464,560, 3,600,306, 3,667,607, 3,902,847, 4,144,306 and 4,258,001. It can contain, in dry form, the various reagents required for the function of the test element. The solvent application zone can be a piece of fabric paper which is located at one end of the test element and which becomes completely saturated with a definite volume of liquid merely by being dipped into a solution, for example a solution of the sample, or by being briefly flushed with tap water, and then releases the liquid to the succeeding zones more slowly and in a controlled manner. The solvent application zone has dimensions such that it takes up sufficient liquid to permit the latter to migrate to the other end of the device, the end of the absorption zone.

Between the solvent application zone and the absorption zone there are located the functional regions in which are contained reaction components for the performance of the test and in which all the reaction stages of the performance of the test take place. Part of the reaction components for the performance of the test can also be housed in the sample application zone. The absorption zone has the function of absorbing excess and freely mobile reagent components and reaction products of the single-producing system.

The absorbent supporting materials in the form of one or more strips, as constituents of the various functional regions, can, according to choice, be composed of cellulose, of chemical derivatives of cellulose or of plastics having a porous or fibrous structure and adequately hydrophilic properties, or of particles such as cellulose or silica gel embedded in a synthetic membrane, and also of natural products which are hydrophilc but have been rendered insoluble in water. A combination of strips composed of different materials can be used. Suitable absorbent materials are selected on the basis of the requirements set for the particular diagnostic device.

Reactants with immunological binding properties such as antigens, haptens or antibodies are incorporated in various embodiments of the device. In the event that glycoproteins or oligosaccharides which attach themselves to lectins are to be detected, one reactant having biological affinity can be the specific lectin, while the second reactant having immunological affinity can be an antibody which is directed against a point of attachment on the analyte other than that of the lectin. In the event that microbial active compounds are to be detected, One combination partner can be the receptor substance for the active compound, while the second combination partner can be an antibody which is directed against another point of attachment on the active compound.

One combination partner having biological affinity becomes attached during the progress of the reaction, or has already been attached to the supporting material in the functional region designed for the detection of the analyte (solid phase zone). It is also called the solid phase combination partner. The other combination partner(s) are present in the supporting materials. They are provided with a labelling.

Amongst the various known possibilities of labelling, enzyme labelling is preferred. It requires chromogenic substrate systems or substrate systems which produce fluorescence or chemiluminescence. Chemiluminescence labelling represents a further example of a labelling which is only measured after the addition of a reagent. It is possible to measure either the chemiluminescence itself or a fluorescence excited by the latter. In most cases fluorescence labelling is measured without the addition of a reagent being required. However, as in the use of certain rare earth chelates, it can also be desirable to produce the fluorophore to be measured only as the result of adding a reagent, or to add a second fluorophore which becomes excited by the first or which excites the first fluorophore. The fluorescence can be measured at one point, as a function of time or as fluorescence polarization.

A reagent required for detection can be induced to react with the immune complex to be detected in various ways, after the separation stage. Part of the signal-producing system can be located in the sold phase zone. After the solid phase has been adequately washed, a reagent required to detect the labelling can be released at a retarded rate in various embodiments in the heterogeneous immunoassay with detection in the bound phase. The following are possible examples:

The application of reagents by means of a stream of liquid arranged parallel to the main stream of liquid, but flowing more slowly and starting from the mobile phase reservoir and entering in front of the zone containing the labelled component. The parallel stream of liquid can be controlled by using an absorbent medium which chromatographs more slowly, for example a paper which chromatographs suitably slowly or a paper which is impregnated in places with "components temporarily blocking the way", such as, for example, polymers which impart a high viscosity on passing into solution (for example polyvinyl alcohols or dextrans).

After the solid phase has been adequately washed (=completion of chromatography), the application of reagents can be effected by pressing down an element which is a solid constituent of the test element. The "pressing down" can be effected mechanically or by removing distance pieces by the action of a stream of liquid. For example, the mechanical pressing down of an element containing the reagents can be effected by pressing down a flap or a piece of paper supported by distance pieces. The lowering of an element containing the reagents by the action of the stream of liquid can be effected, for example, by laminating over one another the solid phase, a water-soluble polymer and the reagent carrier (for example a suitably impregnated piece of paper).

A retarded introduction of reagents into the liquid stream can be effected using a microencapsulated reagent which only emerges from the encapsulation after the solid phase has been adequately washed, or by coating the reagent adhering in the matrix with components which dissolve slowly.

One possible means presented for the special case of enzyme labelling is as follows: when a peroxidase labelling is used, a glucose oxidase zone can be placed in front of the solid phase zone. Glucose and also the chromogen are then incorporated into the liquid stream, which can result in color formation behind the glucose oxidase. Appreciable color formation is only observed

if, at an appropriately high concentration of peroxidase, sufficient H_2O_2 is formed by the oxidase. This formation of the peroxide sets in slowly, reaches an optimum concentration and finally reaches a high concentration which results in inhibition of the enzyme and thus automatic cessation of the color formation. This coloration can be moderated if an H_2O_2-acceptor, for example a thioether as a mild reducing agent, or the enzyme catalase is incorporated in the oxidase zone or in front of the latter.

In this example a reagent for detecting the labelling is produced by a delay circuit, making use of an enzyme. The color formation in the solid phase zone only begins after this zone has been adequately washed free from nonspecifically bound labelling by the stream of liquid.

There are several possible means of preparing the solid phase zone. The components fixed there can be attached by chemical covalent bonds or adsorptively to an absorptive support which is a part of the test element. These components can also be attached to a dispersion of particles which remain fixed at the place of application after they have been applied to an absorbent support. For example, suspensions of cells carrying specific receptors on their surface, such as, for instance, Staphylococcus aureus Cowan I cells, or latex particles carrying combination partners of biological affinity attached to their surface, are suitable for being fixed in a paper matrix. The components of the test strip which are attached to pipettable supports and also the unattached components of the device can be dried onto the absorbent matrix of the element by air drying; freeze-drying stages are not absolutely necessary.

A few test performance will be illustrated as examples of embodiments which can be regarded as independent of the labelling used. For the sake of simplicity, they are only described for the detection of a single analyte by means of the diagnostic device.

The following two embodiments, which conform to the principle of competitive immunoassay, will be described for the case where the analyte has only a single combination point of biological affinity or only one combination point of biological affinity out of several is utilized:

The solid phase combination partner is attached by covalent bonds or adsorptively to the supporting material of the solid phase functional region. The solution of analyte renders mobile a predetermined amount of labelled analyte contained in the diagnostic agent. The two components migrate into the functional sector containing the solid phase combination partner and compete for combination with the solid phase combination partner. If the proportion of analyte is high compared with the labelled analyte, little labelled analyte will be attached. If it is low, a great deal of labelled analyte will be attached.

The solid phase combination partner is housed as an unattached component in a functional region in front of the solid phase functional region. The oncoming front of solvent transports it into the solid phase functional region, where it becomes attached. This solid phase attachment is produced by combination systems of biological affinity which are independent of the combination system of the analyte. A combination partner which is conjugated with biotin attaches itself to avidin attached to the support. An immunoglobulin, such as IgG, as a combination partner, is fixed via its Fc component to support-attached protein A of S. aureus, or is attached by solid phase antibody of another species, non idiotypically directed to said immunoglobulin.

As previously described, the analyte and the labelled analyte compete, as constituents of the diagnostic agent, for the attachments to the solid phase combination partner during the processing period. This competition reaction takes place partly with the dissolved solid phase combination partner and partly with the solid phase combination partner which has already been attached to the solid phase.

If two combination points of differing specificity are present in an analyte, several embodiments, conforming to the principle of sandwich immunoassay, of the diagnostic agent are conceivable. Two of these will also be illustrated below:

If the solid phase combination partner is attached by covalent bonds or adsorptively to the supporting material of the solid phase functional region, the analyte forms, with the labelled combination partner, a binary complex which migrates together with the solvent into the solid phase functional region and reacts there with the solid phase combination partner, with the formation of a ternary complex, attached to the solid phase, which can be detected via the labelling of the first combination partner. The excess labelled combination partner is removed by the solvent into the subsequent functional region, the absorption zone.

If the solid phase combination partner is present in a non-attached form in the diagnostic agent and is rendered mobile by the solvent, the two reactants of the analyte of biological affinity are housed in the functional regions in such a way that the analyte reacts simultaneously or successively with both reactants and the resulting ternary complex then migrates into the solid phase functional region, where, as already described above, it becomes attached to the solid phase via a second system of biological affinity which is independent of that of the analyte.

In order to illustrate the embodiments descried above and further embodiments which conform to the immunometric test principle, the principle of indirect antibody detection or the ELA (enzyme-labelled-antigen) principle of immunoassay, FIGS. 1 and 2 illustrate in an exemplary manner the distribution of the components of the agent in the functional regions and, after the performance of the reaction, the composition of the solid phase complex, the amount of which is a measure of the concentration of analytes in the sample.

It has been found that a completely integrated test strip operating in accordance with the principle of heterogeneous immunoassay by means of solid phase detection is not only feasible in principle, but can, in addition, also be evaluated within a period of less than one hour, the quantification and the sensitivity of conventional RIAs or ELISAs being achieved. The detection of trace components in the range of 10^{-12} mol/liter has been made possible at reaction times of less than 30 minutes, at room temperature, the amounts of sample required being 10^{-16} mol, corresponding, for example, to approx. 1 pg. The arrangements described also enable tests of lower sensitivity requirements to be carried out, however. Standard curves over two to three decades were obtained when evaluation was carried out with the Sanoquell reflectometer (made by Quelle). The chromatography time for the test element, including complete color development, is not more than 16 minutes. Evaluation can also be carried out visually. With HCG as analyte, the start of the range of determination

in an example using a glucose oxidase attached to a solid phase and a peroxidase labelling was 0.3 ng/ml (corresponding to 3 U/liter).

In the example following, the application of the principle of the competitive double antibody test is presented as a concrete embodiment. In this test configuration, four components have to be reacted successively for the determination reaction and the separation stage, the reaction times and the concentrations of the reactant being critical values. The example is not to be regarded as limiting in any way, but merely serves to illustrate the subject of the invention further.

EXAMPLE

Completely integrated enzyme-immunochemical device for the detection of HCG by means of a built-in chromogen substrate system.

1.1. Reagents

1.1.1. HCG-peroxidase conjugate

HCG having a specific activity of approx. 3000 U/mg was obtained from Organon. Peroxidase from horseradish was obtained from Boehringer Mannheim (catalog no. 413,470). The hetero-bifunctional reagent N-γ-maleimidobutyryloxysuccinimide (GMBS) was obtained from Behring Diagnostics and was reacted with the HCG as described by Tranimori et al., 1983, in J.Imm. Meth. 62, 123–131. 2-iminothiolane hydrochloride (Sigma, catalog no. I 6256) was reacted with peroxidase as described by King et al., 1978, in Biochemistry 17, 1499–1506. A conjugate was prepared from the GMBS-HCG and the iminothiolane-peroxidase as described by Tanimori et al. The crude conjugate was purified by gel chromatography over Ultrogel ACA 44 (LKB). The fraction in which about 1–2 peroxidase molecules were coupled per HCG molecule was used for the test. The conjugate was diluted with Enzygnost IgE incubation medium made by Behringwerke, order no. OS D, designated briefly as incubation medium in the following text.

1.1.2. Antibodies

Antibodies against HCG were obtained by immunizing rabbits, and antibodies against rabbit-IgG were obtained by immunizing goats. The IgG fractions were isolated from serum by ammonium sulfate precipitation and anion exchange chromatography, and were purified further by immunadsorption. The methods used are described in the book "Immunologische Arbeitsmethoden" (Immunological working methods), Helmut Friemel, Editor, 1984, Gustav Fischer Verlag, Stuttgart. The anti-HCG antibody was finally diluted in the conjugate dilution buffer indicated above.

1.1.3. Glucose oxidase

Glucose oxidase from Aspergillus niger was obtained as a solution containing 300 U/mg (Serva, catalog No. 22,737). The glucose oxidase was finally diluted with incubation medium.

1.1.4. Glucose and Tetramethylbenzidine

α-D-glucose and tetramethylbenzidine hydrochloride were obtained from Serva, catalog no. 22,720 and 35,926, respectively.

1.2. Preparation of the Device

The sheet-like functional regions were prepared as follows:

The mobile phase application zone was prepared by cutting, to dimensions of 20×6 mm, a fabric sponge cloth made by Kalle; this is a synthetic sponge of regenerated cellulose which has been compressed in a dry state. It was impregnated with a solution of 50 mg of glucose and 0.75 mg of tetramethylbenzidine hydrochloride per ml of water, and was dried in a stream of air.

The conjugate, the anti-HCG antibody and glucose oxidase (5 µl of each at 25 µl/ml, 100 µl/ml and 0.1 mg/ml, respectively) were applied behind one another, at uniform distance, to a 45×5 mm piece of MN no. 1 paper (Macherey & Nagel), and were dried in the air.

A piece measuring 5×5 mm of Schleicher & Schull No. 597 paper was coated in a covalent manner with anti-rabbit IgG-antibody as the solid phase zone. This was effected by coupling the antibody with the paper, which had been activated with cyanogen bromide. as described by Clarke et al., 1979, Meth.Enzymology, volume 68, 441–442.

A 20×5 mm piece of Schleicher & Schüll No. 2668/8 paper was used as the absorption zone.

The four pieces of paper, with a 0.5–1 mm overlap behind one another, were fixed on a plastic ribbon by means of double-sided adhesive tape (Tesaband made by Beiersdorf), so that a test strip 5 mm wide was formed.

1.3. Performance of the Test

The test was carried out in each case by applying 200 µl of an HCG dilution in incubation medium to the fabric.

1.4 Results

The chromatographic development of the test element and the self-actuating color development were complete after 15 minutes at room temperature, and evaluation could be carried out either visually or by means of a reflectometer.

The following values were obtained when evaluating the solid phase zone (No. 597 paper) with the Sanoquell blood glucose evaluation apparatus made by Quelle:

HCG concentration (U/liter)	Measured values (mg of glucose per dl of blood)
0.3	107
3	117
30	95
300	70
3000	0

The following values were obtained with the same test strips using the Rapimat urine test strip evaluation apparatus made by Behringwerke:

HCG concentration (U/liter)	Measured values (BIT)
0.3	76
3	76
30	94
300	119